

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C07K 14/35 // G01N 33/53</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 96/38478</b> <b>(43) International Publication Date:</b> 5 December 1996 (05.12.96)
<b>(21) International Application Number:</b> PCT/SE96/00319 <b>(22) International Filing Date:</b> 12 March 1996 (12.03.96)  <b>(30) Priority Data:</b> 9501976-6 30 May 1995 (30.05.95) SE 9502596-1 13 July 1995 (13.07.95) SE 9503246-2 19 September 1995 (19.09.95) SE  <b>(71) Applicant (for all designated States except US):</b> ASTRA AKTIEBOLAG [SE/SE]; S-151 85 Södertälje (SE).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> BALGANESH, Meenakshi [IN/IN]; 47, 5th Main, Postal Colony 2nd Stage, Sanjaynagar, Bangalore 560094 (IN). SHARMA, Umender [IN/IN]; House No. 385, 12th 'C' Cross, Vyalikaval, Bangalore 560003 (IN).  <b>(74) Agent:</b> ASTRA AKTIEBOLAG; Patent Dept., S-151 85 Södertälje (SE).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> NEW DNA MOLECULES  <b>(57) Abstract</b>  The present invention provides novel nucleic acid molecules coding for sigma subunits of <i>Mycobacterium tuberculosis</i> RNA polymerase. It also relates to polypeptides, referred to as SigA and SigB, encoded by such nucleic acid molecules, as well as to vectors and host cells transformed with the said nucleic acid molecules. The invention further provides screening assays for compounds which inhibit the interaction between a sigma subunit and a core RNA polymerase.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

## NEW DNA MOLECULES

### TECHNICAL FIELD

5     The present invention provides novel nucleic acid molecules coding for sigma subunits of *Mycobacterium tuberculosis* RNA polymerase. It also relates to polypeptides, referred to as SigA and SigB, encoded by such nucleic acid molecules, as well as to vectors and host cells transformed with the said nucleic acid molecules. The invention further provides  
10    screening assays for compounds which inhibit the interaction between a sigma subunit and a core RNA polymerase.

### BACKGROUND ART

15    Transcription of genes to the corresponding RNA molecules is a complex process which is catalyzed by DNA dependent RNA polymerase, and involves many different protein factors. In eubacteria, the core RNA polymerase is composed of  $\alpha$ ,  $\beta$ , and  $\beta'$  subunits in the ratio 2:1:1. To  
20    direct RNA polymerase to promoters of specific genes to be transcribed, bacteria produce a variety of proteins, known as sigma ( $\sigma$ ) factors, which interact with RNA polymerase to form an active holoenzyme. The resulting complexes are able to recognize and attach to selected nucleotide sequences in promoters.

25    Physical measurements have shown that the sigma subunit induces conformational transition upon binding to the core RNA polymerase. Binding of the sigma subunit to the core enzyme increases the binding constant of the core enzyme for DNA by several orders of magnitude  
30    (Chamberlin, M.J. (1974) Ann. Rev. Biochem. 43, 721-).

Characterisation of sigma subunits, identified and sequenced from various organisms, allows them to be classified into two broad categories; Group I and Group II. The Group I sigma has also been referred to as the sigma<sup>70</sup> class, or the "house keeping" sigma group. Sigma subunits belonging to  
5 this group recognise similar promoter sequences in the cell. These properties are reflected in certain regions of the proteins which are highly conserved between species.

Bacterial sigma factors do not have any homology with eukaryotic  
10 transcription factors, and are consequently a potential target for antibacterial compounds. Mutations in the sigma subunit, effecting its association and ability to confer DNA sequence specificity to the enzyme, are known to be lethal to the cell.

15 *Mycobacterium tuberculosis* is a major pulmonary pathogen which is characterized by its very slow growth rate. As a pathogen it gains access to alveolar macrophages where it multiplies within the phagosome, finally lysing the cells and being disseminated through the blood stream, not only to other areas of the lung, but also to extrapulmonary tissues. Thus the  
20 pathogen multiplies in at least two entirely different environments, which would involve the utilisation of different nutrients and a variety of possible host factors; a successful infection would thus involve the coordinated expression of new sets of genes. This regulation would resemble different physiological stages, as best exemplified by *Bacillus*, in which the  
25 expression of genes specific for different stages are transcribed by RNA polymerases associating with different sigma factors. This provides the possibility of targeting not only the house keeping sigma of *M. tuberculosis*, but also sigma subunits specific for the different stages of infection and dissemination.

## BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1: Map of plasmid pARC 8175

Fig. 2: Map of plasmid pARC 8176

5

## PURPOSE OF THE INVENTION

10 Since the association to a specific sigma subunit is essential for the specificity of RNA polymerase, this process of association is a suitable target for drug design. In order to identify compounds capable of inhibiting the said association process, the identification of the primary structures of sigma subunits is desirable.

15 It is thus the purpose of the invention to provide information on sequences and structure of sigma subunits, which information will enable the screening, identification and design of compounds competing with the sigma subunit for binding to the core RNA polymerase, which compounds may be developed into effective therapeutic agents.

20

## DISCLOSURE OF THE INVENTION

25 Throughout this description and in particular in the following examples, the terms "standard protocols" and "standard procedures", when used in the context of molecular cloning techniques, are to be understood as protocols and procedures found in an ordinary laboratory manual such as: Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A laboratory manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold  
30 Spring Harbor, NY.

In a first aspect, this invention provides an isolated polypeptide which is a Group I sigma subunit of *Mycobacterium tuberculosis* RNA polymerase, or a functionally equivalent modified form thereof.

- 5 Preferred such polypeptides having amino acid sequences according to SEQ ID NO: 2 or 4 of the Sequence Listing have been obtained by recombinant DNA techniques and are hereinafter referred to as SigA and SigB polypeptides. However, it will be understood that the polypeptides according to the invention are not limited strictly to polypeptides with an
- 10 amino acid sequence identical with SEQ ID NO: 2 or 4 in the Sequence Listing. Rather the invention additionally encompasses modified forms of these native polypeptides carrying modifications like substitutions, small deletions, insertions or inversions, which polypeptides nevertheless have substantially the biological activities of a *M. tuberculosis* sigma subunit.
- 15 Such biological activities comprise the ability to associate with the core enzyme and / or confer the property of promoter sequence recognition and initiation of transcription. Included in the invention are consequently polypeptides, the amino acid sequence of which are at least 90% homologous, preferably at least 95% homologous, with the amino acid
- 20 sequence shown as SEQ ID NO: 2 or 4 in the Sequence Listing.

- In another aspect, the invention provides isolated and purified nucleic acid molecules which have a nucleotide sequence coding for a polypeptide of the invention e.g. the SigA or SigB polypeptide. In a preferred form of the
- 25 invention, the said nucleic acid molecules are DNA molecules which have a nucleotide sequence identical with SEQ ID NO: 1 or 3 of the Sequence Listing. However, the nucleic acid molecules according to the invention are not to be limited strictly to the DNA molecules with the sequence shown as SEQ ID NO: 1 or 3. Rather the invention encompasses nucleic acid
- 30 molecules carrying modifications like substitutions, small deletions, insertions or inversions, which nevertheless encode proteins having substantially the biochemical activity of the polypeptides according to the

invention. Included in the invention are consequently DNA molecules, the nucleotide sequences of which are at least 90% homologous, preferably at least 95% homologous, with the nucleotide sequence shown as SEQ ID NO: 1 or 3 in the Sequence Listing.

5

Included in the invention are also DNA molecule which nucleotide sequences are degenerate, because of the genetic code, to the nucleotide sequences shown as SEQ ID NO: 1 or 3. A sequential grouping of three nucleotides, a "codon", codes for one amino acid. Since there are 64 possible codons, but only 20 natural amino acids, most amino acids are coded for by more than one codon. This natural "degeneracy", or "redundancy", of the genetic code is well known in the art. It will thus be appreciated that the DNA sequence shown in the Sequence Listing is only an example within a large but definite group of DNA sequences which will encode the polypeptide as described above.

10  
15

Included in the invention are consequently isolated nucleic acid molecule selected from:

(a) DNA molecules comprising a nucleotide sequence as shown in SEQ ID NO: 1 or SEQ ID NO: 3 encoding a Group I sigma subunit of *Mycobacterium tuberculosis* RNA polymerase;

20

(b) nucleic acid molecules comprising a nucleotide sequence capable of hybridizing to a nucleotide sequence complementary the polypeptide coding region of a DNA molecule as defined in (a) and which codes for a polypeptide which is a Group I sigma subunit of *Mycobacterium tuberculosis* or a functionally equivalent modified form thereof; and

25

(c) nucleic acid molecules comprising a nucleic acid sequence which is degenerate, as a result of the genetic code, to a nucleotide sequence as defined in (a) or (b) and which codes for a polypeptide which is a Group I sigma subunit of *Mycobacterium tuberculosis* or a functionally equivalent modified form thereof.

30

The term "hybridizing to a nucleotide sequence" should be understood as hybridizing to a nucleotide sequence, or a specific part thereof, under stringent hybridization conditions which are known to a person skilled in the art.

5

A DNA molecule of the invention may be in the form of a vector, e.g. a replicable expression vector which carries and is capable of mediating the expression of a DNA molecule according to the invention. In the present context the term "replicable" means that the vector is able to replicate in a given type of host cell into which it has been introduced. Examples of  
10 vectors are viruses such as bacteriophages, cosmids, plasmids and other recombination vectors. Nucleic acid molecules are inserted into vector genomes by methods well known in the art. Vectors according to the invention can include the plasmid vector pARC 8175 (NCIMB 40738) which  
15 contains the coding sequence of the *sigA* gene, or pARC 8176 (NCIMB 40739) which contains the coding sequence of the *sigB* gene.

Included in the invention is also a host cell harbouring a vector according to the invention. Such a host cell can be a prokaryotic cell, a unicellular  
20 eukaryotic cell or a cell derived from a multicellular organism. The host cell can thus e.g. be a bacterial cell such as an *E. coli* cell; a cell from a yeast such as *Saccharomyces cerevisiae* or *Pichia pastoris*, or a mammalian cell. The methods employed to effect introduction of the vector into the host cell are standard methods well known to a person familiar with  
25 recombinant DNA methods.

A further aspect of the invention is a process for production of a polypeptide of the invention, comprising culturing host cells transformed with an expression vector according to the invention under conditions  
30 whereby said polypeptide is produced, and recovering said polypeptide.



The medium used to grow the cells may be any conventional medium suitable for the purpose. A suitable vector may be any of the vectors described above, and an appropriate host cell may be any of the cell types listed above. The methods employed to construct the vector and effect  
5 introduction thereof into the host cell may be any methods known for such purposes within the field of recombinant DNA. The recombinant polypeptide expressed by the cells may be secreted, i.e. exported through the cell membrane, dependent on the type of cell and the composition of the vector.

10

If the polypeptide is produced intracellularly by the recombinant host, i.e. is not secreted by the cell, it may be recovered by standard procedures comprising cell disruption by mechanical means, e.g. sonication or homogenization, or by enzymatic or chemical means followed by  
15 purification.

In order to be secreted, the DNA sequence encoding the polypeptide should be preceded by a sequence coding for a signal peptide, the presence of which ensures secretion of the polypeptide from the cells so that at least  
20 a significant proportion of the polypeptide expressed is secreted into the culture medium and recovered.

Another important aspect of the invention is a method of assaying for compounds which have the ability to inhibit the association of a sigma  
25 subunit to a *Mycobacterium tuberculosis* RNA polymerase, said method comprising the use of a recombinant SigA or SigB polypeptide or a nucleic acid molecule as defined above. Such a method will preferably comprise (i) contacting a compound to be tested for such inhibition ability with a SigA or SigB polypeptide as described above and a *Mycobacterium tuberculosis*  
30 core RNA polymerase; and (ii) detecting whether the said polypeptide associates with the said core RNA polymerase to form RNA polymerase holoenzyme. The term "core RNA polymerase" is to be understood as an

RNA polymerase which comprises at least the  $\alpha$ ,  $\beta$ , and  $\beta'$  subunits, but not the sigma subunit. The term "RNA polymerase holoenzyme" is to be understood as an RNA polymerase comprising at least the  $\alpha$ ,  $\beta$ ,  $\beta'$  and sigma subunits. If desirable, the sigma subunit polypeptide can be labelled,  
5 for example with a suitable radioactive molecule, e.g.  $^{35}\text{S}$  or  $^{125}\text{I}$ .

Suitable methods for determining whether a sigma polypeptide has associated to core RNA polymerase are disclosed by Lesley et al. (Biochemistry 28, 7728-7734, 1989). Such a method may thus be based on  
10 the size difference between sigma polypeptides bound to core RNA polymerase, versus polypeptides not bound. This difference in size allows the two forms to be separated by chromatography, e.g. on a gel filtration column, such as a Waters Protein Pak<sup>®</sup> 300SW sizing column. The two forms eluted from the column may be detected and quantified by known  
15 methods, such as scintillation counting or SDS-PAGE followed by immunoblotting.

According to another method also described by Lesley et al. (*supra*), RNA polymerase holoenzyme is detected by immunoprecipitation using an  
20 antibody binding to RNA polymerase holoenzyme. Core RNA polymerase from an organism such as *E. coli*, *M. tuberculosis* or *M. smegmatis* can be allowed to react with a radiolabelled SigA or SigB polypeptide. The reaction mix is treated with *Staphylococcus aureus* formalin-treated cell suspension, pretreated with an anti-RNA polymerase antibody. The cell  
25 suspension is washed to remove unbound proteins, resuspended in SDS-PAGE sample buffer and separated on SDS-PAGE. Bound SigA or SigB polypeptides are monitored by autoradiography followed by scintillation counting.

30 Another method of assaying for compounds which have the ability to inhibit sigma subunit-dependent transcription by a *Mycobacterium tuberculosis* RNA polymerase can comprise (i) contacting a compound to be

- tested for said inhibition ability with a polypeptide of the invention, a *Mycobacterium tuberculosis* core RNA polymerase, and a DNA having a coding sequence operably-linked to a promoter sequence capable of recognition by said core RNA polymerase when bound to said polypeptide, said contacting being carried out under conditions suitable for transcription of said coding sequence when *Mycobacterium tuberculosis* RNA polymerase is bound to said promoter; and (ii) detecting formation of mRNA corresponding to said coding sequence.
- 10 Such an assay is based on the fact that *E. coli* consensus promoter sequences are not transcribable by core RNA polymerase lacking the sigma subunit. However, addition of a sigma<sup>70</sup> protein will enable the complex to recognise specific promoters and initiate transcription. Screening of compounds which have the ability to inhibit sigma-dependent transcription can thus be performed, using DNA containing a suitable promoter as a template, by monitoring the formation of mRNA of specific lengths. Transcription can be monitored by measuring incorporation of <sup>3</sup>H-UTP into TCA-precipitable counts (Ashok Kumar et al. (1994) J. Mol. Biol. 235, 405-413; Kajitani, M. and Ishihama, A. (1983) Nucleic Acids Res. 11, 671-686 and 3873-3888) and determining the length of the specific transcript. Compounds which are identified by such an assay can inhibit transcription by various mechanisms, such as (a) binding to a sigma protein and preventing its association with the core RNA polymerase; (b) binding to core RNA polymerase and sterically inhibiting the binding of a sigma protein; or (c) inhibiting intermediate steps involved in the initiation or elongation during transcription.
- 30 A further aspect of the invention is a method of determining the protein structure of a *Mycobacterium tuberculosis* RNA polymerase sigma subunit, characterised in that a SigA or SigB polypeptide is utilized in X-ray crystallography. The use of SigA or SigB polypeptide in crystallisation will facilitate a rational design, based on X-ray crystallography, of therapeutic

5

EXAMPLE 1: Identification of *M. tuberculosis* DNA sequences homologous to the sigma<sup>70</sup> gene

10

The following PCR primers were designed, based on the conserved amino acid sequences of sigma<sup>45</sup> (a sigma<sup>70</sup> homologue) of *Bacillus subtilis* and sigma<sup>70</sup> of *E. coli* (Gitt, M.A. et al. (1985) J. Biol. Chem. 260, 7178-7185):

15

5'-AAG TTC AGC ACG TAC GCC ACG TGG TGG ATC-3'  
                  C          G      C

20

5'-CTT GGC CTC GAT CTG GCG GAT GCG CTC-3.  
C C C

25

30 Chromosomal DNA from *M. tuberculosis* H37RV (ATCC 27294) was prepared following standard protocols. PCR amplification of a DNA fragment of approximately 500 bp was carried out using the following conditions:

30

-11-

Annealing:	+55°C	1 min
Denaturation:	+93°C	1 min
Extension:	+73°C	2 min

5     1.2. Southern hybridisation of *M. tuberculosis* DNA

Chromosomal DNA from *M. tuberculosis* H37RV (ATCC 27294),  
*M. tuberculosis* H37RA and *Mycobacterium smegmatis* was prepared  
following standard protocols and restricted with the restriction enzyme  
10     *Sal*I. The DNA fragments were resolved on a 1% agarose gel by  
electrophoresis and transferred onto nylon membranes which were  
subjected to "Southern blotting" analysis following standard procedures. To  
detect homologous fragments, the membranes were probed with a  
radioactively labelled ~500 bp DNA fragment, generated by PCR as  
15     described above.

Analysis of the Southern hybridisation experiment revealed the presence of  
at least three hybridising fragments of approximately 4.2, 2.2 and 0.9 kb,  
respectively, in the *Sal*I-digested DNA of both of the *M. tuberculosis* strains.  
20     In *M. smegmatis*, two hybridising fragments of 4.2 and 2.2 kb, respectively,  
were detected. It could be concluded that there were multiple DNA  
fragments with homology to the known sigma<sup>70</sup> genes.

Similar Southern hybridisation experiments, performed with four different  
25     clinical isolates of *M. tuberculosis*, revealed identical patterns, indicating the  
presence of similar genes also in other virulent isolates of *M. tuberculosis*.

EXAMPLE 2: Cloning of putative sigma<sup>70</sup> homologues

30

2.1. Cloning of *M. tuberculosis* sigA

A lambda gt11 library (obtained from WHO) of the chromosomal DNA of *M. tuberculosis* Erdman strain was screened, using the 500 bp PCR probe as described above, following standard procedures. One lambda gt11 phage with a 4.7 kb *EcoRI* insert was identified and confirmed to hybridise with the PCR probe. Restriction analysis of this 4.7 kb insert revealed it to have an internal 2.2 kb *SalI* fragment which hybridised with the PCR probe.

The 4.7 kb fragment was excised from the lambda gt 11 DNA by *EcoRI* restriction, and subcloned into the cloning vector pBR322, to obtain the recombinant plasmid pARC 8175 (Fig. 1) (NCIMB 40738).

The putative sigma<sup>70</sup> homologue on the 2.2 kb *SalI* fragment was designated *M. tuberculosis sigA*. The coding sequence of the *sigA* gene was found to have an internal *SalI* site, which could explain the hybridisation of the 0.9 kb fragment in the Southern experiments.

## 2.2. Cloning of *M. tuberculosis sigB*

*M. tuberculosis* H37Rv DNA was restricted with *SalI* and the DNA fragments were resolved by preparative agarose gel electrophoresis. The agarose gel piece corresponding to the 4.0 to 5.0 kb size region was cut out, and the DNA from this gel piece was extracted following standard protocols. This DNA was ligated to the cloning vector pBR329 at its *SalI* site, and the ligated DNA was transformed into *E. coli* DH5 $\alpha$  to obtain a sub-library. Transformants of this sub-library were identified by colony blotting, using the PCR-derived 500 bp probe, following standard protocols. Individual transformant colonies were analyzed for their plasmid profile. One of the recombinant plasmids retaining the expected plasmid size, was analyzed in detail by restriction mapping and was found to harbour the expected 4.2 kb *SalI* DNA fragment. This plasmid with the *sigB* gene on the 4.2 kb insert was designated pARC 8176 (Fig. 2) (NCIMB 40739).

EXAMPLE 3: Nucleotide sequence of *M. tuberculosis sigA* and *sigB* genes3.1. Nucleotide sequence of *sigA*

5 The *EcoRV* - *EcoRI* DNA fragment expected to encompass the entire *sigA* gene was subcloned into appropriate M13 vectors and both strands of the gene sequenced by the dideoxy method. The sequence obtained is shown as SEQ ID NO: 1 in the Sequence Listing. An open reading frame (ORF) of 1580 nucleotides (positions 70 to 1650 in SEQ ID NO: 1) coding for a  
10 protein of 526 amino acids was predicted from the DNA sequence. The N-terminal amino acid has been assigned tentatively based on the first GTG (initiation codon) of the ORF.

The derived amino acid sequence of the gene product SigA (SEQ ID NO: 2) showed 60% identity with the *E. coli* sigma<sup>70</sup> and 70% identity with the HrdB sequence of *Streptomyces coelicolor*. The overall anatomy of the SigA sequence is compatible with that seen among sigma<sup>70</sup> proteins of various organisms. This anatomy comprises a highly conserved C-terminal half, while the N-terminal half generally shows lesser homology. The two  
20 regions are linked by a stretch of amino acids which varies in length and is found to be generally unique for the protein. The SigA sequence has a similar structure, where the unconserved central stretch correspond to amino acids 270 to 306 in SEQ ID NO: 2.

25 The N-terminal half has limited homology to *E. coli* sigma<sup>70</sup>, but shows resemblance to that of the sigma<sup>70</sup> homologue HrdB of *S. coelicolor*. The highly conserved motifs of regions 3.1, 3.2, 4.1 and 4.2 of *S. coelicolor* which were proposed to be involved in DNA binding (Lonetto, M. et al. (1992) J. Bacteriol. 174, 3843-3849) are found to be nearly identical also in the  
30 *M. tuberculosis* SigA sequence. The N-terminal start of the protein has been tentatively assigned, based on homologous motifs of the *S. coelicolor* HrdB sequence.

The overall sequence similarity of the SigA and SigB amino acid sequences to known sigma<sup>70</sup> sequences suggests assignment of the *M. tuberculosis* SigA to the Group I sigma<sup>70</sup> proteins. However, SigA also shows distinct differences with known sigma<sup>70</sup> proteins, in particular a unique and  
5 lengthy N-terminal stretch of amino acids (positions 24 to 263 in SEQ ID NO: 2), which may be essential for the recognition and initiation of transcription from promoter sequences of *M. tuberculosis*.

### 3.2. Nucleotide sequence of *sigB*

10 The nucleotide sequence of the *sigB* gene (SEQ ID NO: 3) encodes a protein of 323 amino acids (SEQ ID NO: 4). The N-terminal start of the protein has been tentatively identified based on the presence of the first methionine of the ORF. The ORF is thus estimated to start at position 325 and to end at  
15 1293 in SEQ ID NO: 3. Alignment of the amino acid sequence of the *sigB* gene with other sigma<sup>70</sup> proteins places the *sigB* gene into the Group I family of sigma<sup>70</sup> proteins. The overall structure of the gene product SigB follows the same pattern as described for SigA. However, the SigB  
20 sequence has only 60% homology with the SigA sequence, as there are considerable differences not only within the unconserved regions of the protein, but also within the putative DNA binding regions of the *sigB* protein. These characteristics suggest that the SigB protein may play a distinct function in the physiology of the organism.

25

### EXAMPLE 4: Expression of *sigA* and *sigB*

#### 4.1. Expression of *M. tuberculosis sigA* gene in *E. coli*

30 The N-terminal portion of the *sigA* gene was amplified by PCR using the following primers:



5

5'-GTA CAG GCC AGC CTC GAT CCG CTT GGC-3'

- 30

The plasmid pARC 8171 was transformed into the T<sub>7</sub> expression host *E. coli* BL21(DE3). Individual transformants were screened for the presence of the 6.35 kb plasmid and confirmed by restriction analysis. One of the

- transformants was grown at 37°C and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) using standard protocols. A specific 90 kDa protein was induced on expression. Cells were harvested by low speed centrifugation and lysed by sonication in phosphate buffered saline, pH 7.4. The lysate was centrifuged at 100,000 x g to fractionate into supernatant and pellet. The majority of the 70 kDa product obtained after induction with IPTG was present in the pellet fraction, indicating that the protein formed inclusion bodies.
- For purifying the induced *sigA* gene product, the cell lysate as obtained above was clarified by centrifugation at 1000 rpm in Beckman JA 21 rotor for 15 min. The clarified supernatant was layered on a 15-60% sucrose gradient and centrifuged at 100,000 x g for 60 min. The inclusion bodies sedimented as a pellet through the 60% sucrose cushion. This pellet was solubilised in 6 M guanidine hydrochloride which was removed by sequential dialysis against buffer containing decreasing concentration of guanidine hydrochloride. The dialysate was 75% enriched for the SigA protein which was purified essentially following the protocol for purification *E. coli* sigma<sup>70</sup> as described by Brokhov, S. and Goldfarb, A. (1993) Protein expression and purification, vol. 4, 503-511.

#### 4.2. Expression of *M. tuberculosis sigB* gene in *E. coli*

- The *sigB* gene product was expressed and purified from inclusion bodies. The coding sequence of the *sigB* gene was amplified by PCR using the following primers:

Forward primer (SEQ ID NO: 9), comprising an *NcoI* restriction site:

5'- TTTC ATG GCC GAT GCA CCC ACA AGG GCC-3'  
M A D A P T R A

Reverse primer (SEQ ID NO: 10), comprising an *EcoRI* restriction site:

5'- CTT GAA TTC AGC TGG CGT ACG ACC GCA-3'

The amplified 920 bp fragment was digested with *EcoRI* and *NcoI* and ligated to the *EcoRI*- and *NcoI*-digested pRSET B (Kroll et al. (1993) DNA and Cell Biology 12, 441). The ligation mix was transformed into *E. coli* DH5 $\alpha$ . Individual transformants were screened for plasmid profile and restriction analysis. The recombinant plasmid having the expected plasmid profile was designated pARC 8193.

*E. coli* DH5 $\alpha$  harbouring pARC 8193 was cultured in LB containing in 50  $\mu$ g/ml ampicillin till an OD of 0.5, and induced with 1 mM IPTG at 37°C, following standard protocols. The induced SigB protein was obtained as inclusion bodies which were denatured and renatured following the same protocol as described for the SigA protein. The purified SigB protein was >90% homogenous and suitable for transcription assays.

15

#### DEPOSIT OF MICROORGANISMS

The following plasmids have been deposited under the Budapest Treaty at the National Collections of Industrial and Marine Bacteria (NCIMB), Aberdeen, Scotland, UK.

20

<u>Plasmid</u>	<u>Accession No.</u>	<u>Date of deposit</u>
pARC 8175	NCIMB 40738	15 June 1995
pARC 8176	NCIMB 40739	15 June 1995

25

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

(A) NAME: Astra AB  
 (B) STREET: Västra Mälarehamnen 9  
 (C) CITY: Södertälje  
 (E) COUNTRY: Sweden  
 (F) POSTAL CODE (ZIP): S-151 85  
 (G) TELEPHONE: +46-8-553 260 00  
 (H) TELEFAX: +46-8-553 288 20  
 (I) TELEX: 19237 astra s

(ii) TITLE OF INVENTION: New DNA Molecules

(iii) NUMBER OF SEQUENCES: 10

## (iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
 (B) COMPUTER: IBM PC compatible  
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS  
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1724 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: both  
 (D) TOPOLOGY: linear

## (vi) ORIGINAL SOURCE:

(A) ORGANISM: Mycobacterium tuberculosis  
 (B) STRAIN: Erdman strain

## (vii) IMMEDIATE SOURCE:

(B) CLONE: pARC 8175

## (ix) FEATURE:

(A) NAME/KEY: CDS  
 (B) LOCATION: 70..1653

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AACTAGCAGA CACTTTCGGT TACGCACGCC CAGACCCAAC CGGAAGTGAG TAACGACCGA	60
AGGGTGTAT GTG GCA GCG ACC AAA GCA AGC ACG GCG ACC GAT GAG CCG	108
Val Ala Ala Thr Lys Ala Ser Thr Ala Thr Asp Glu Pro	
1 5 10	
GTA AAA CGC ACC GCC ACC AAG TCG CCC GCG GCT TCC GCG TCC GGG GCC	156
Val Lys Arg Thr Ala Thr Lys Ser Pro Ala Ala Ser Ala Ser Gly Ala	
15 20 25	
AAG ACC GGC GCC AAG CGA ACA GCG GCG AAG TCC GCT AGT GGC TCC CCA	204
Lys Thr Gly Ala Lys Arg Thr Ala Ala Lys Ser Ala Ser Gly Ser Pro	
30 35 40 45	
CCC GCG AAG CGG GCT ACC AAG CCC GCG GCC CGG TCC GTC AAG CCC GCC	252
Pro Ala Lys Arg Ala Thr Lys Pro Ala Ala Arg Ser Val Lys Pro Ala	
50 55 60	

TCG GCA CCC CAG GAC ACT ACG ACC AGC ACC ATC CCG AAA AGG AAG ACC Ser Ala Pro Gln Asp Thr Thr Thr Ser Thr Ile Pro Lys Arg Lys Thr 65 70 75	300
CGC GCC GCG GCC AAA TCC GCC GCC GCG AAG GCA CCG TCG GCC CGC GGC Arg Ala Ala Ala Lys Ser Ala Ala Lys Ala Pro Ser Ala Arg Gly 80 85 90	348
CAC GCG ACC AAG CCA CGG GCG CCC AAG GAT GCC CAG CAC GAA GCC GCA His Ala Thr Lys Pro Arg Ala Pro Lys Asp Ala Gln His Glu Ala Ala 95 100 105	396
ACG GAT CCC GAG GAC GCC CTG GAC TCC GTC GAG GAG CTC GAC GCT GAA Thr Asp Pro Glu Asp Ala Leu Asp Ser Val Glu Glu Leu Asp Ala Glu 110 115 120 125	444
CCA GAC CTC GAC GTC GAG CCC GGC GAG GAC CTC GAC CTT GAC GCC GCC Pro Asp Leu Asp Val Glu Pro Gly Glu Asp Leu Asp Leu Asp Ala Ala 130 135 140	492
GAC CTC AAC CTC GAT GAC CTC GAG GAC GAC GTG GCG CCG GAC GCC GAC Asp Leu Asn Leu Asp Asp Leu Glu Asp Asp Val Ala Pro Asp Ala Asp 145 150 155	540
GAC GAC CTC GAC TCG GGC GAC GAC GAA GAC CAC GAA GAC CTC GAA GCT Asp Asp Leu Asp Ser Gly Asp Asp Glu Asp His Glu Asp Leu Glu Ala 160 165 170	588
GAG GCG GCC GTC GCG CCC GGC CAG ACC GCC GAT GAC GAC GAG GAG ATC Glu Ala Ala Val Ala Pro Gly Gln Thr Ala Asp Asp Asp Glu Glu Ile 175 180 185	636
GCT GAA CCC ACC GAA AAG GAC AAG GCC TCC GGT GAT TTC GTC TGG GAT Ala Glu Pro Thr Glu Lys Asp Lys Ala Ser Gly Asp Phe Val Trp Asp 190 195 200 205	684
GAA GAC GAG TCG GAG GCC CTG CGT CAA GCA CGC AAG GAC GCC GAA CTC Glu Asp Glu Ser Glu Ala Leu Arg Gln Ala Arg Lys Asp Ala Glu Leu 210 215 220	732
ACC GCA TCC GCC GAC TCG GTT CGC GCC TAC CTC AAA CAG ATC GGC AAG Thr Ala Ser Asp Ser Val Arg Ala Tyr Leu Lys Gln Ile Gly Lys 225 230 235	780
GTA GCG CTG CTC AAC GCC GAG GAA GAG GTC GAG CTA GCC AAG CGG ATC Val Ala Leu Leu Asn Ala Glu Glu Glu Val Glu Leu Ala Lys Arg Ile 240 245 250	828
GAG GCT GGC CTG TAC GCC ACG CAG CTG ATG ACC GAG CTT AGC GAG CGC Glu Ala Gly Leu Tyr Ala Thr Gln Leu Met Thr Glu Leu Ser Glu Arg 255 260 265	876
GGC GAA AAG CTG CCT GCC GCC CAG CGC CGC GAC ATG ATG TGG ATC TGC Gly Glu Lys Leu Pro Ala Ala Gln Arg Arg Asp Met Met Trp Ile Cys 270 275 280 285	924
CGC GAC GGC GAT CGC GCG AAA AAC CAT CTG CTG GAA GCC AAC CTG CGC Arg Asp Gly Asp Arg Ala Lys Asn His Leu Leu Glu Ala Asn Leu Arg 290 295 300	972
CTG GTG GTT TCG CTA GCC AAG CGC TAC ACC GGC CGG GGC ATG GCG TTT Leu Val Val Ser Leu Ala Lys Arg Tyr Thr Gly Arg Gly Met Ala Phe 305 310 315	1020
CTC GAC CTG ATC CAG GAA GGC AAC CTG GGG CTG ATC CGC GCG GTG GAG Leu Asp Leu Ile Gln Glu Gly Asn Leu Gly Leu Ile Arg Ala Val Glu 320 325 330	1068

-20-

AAG TTC GAC TAC ACC AAG GGG TAC AAG TTC TCC ACC TAC GCT ACG TGG Lys Phe Asp Tyr Thr Lys Gly Tyr Lys Phe Ser Thr Tyr Ala Thr Trp 335 340 345	1116
TGG ATT CGC CAG GCC ATC ACC CGC GCC ATG GCC GAC CAG GCC CGC ACC Trp Ile Arg Gln Ala Ile Thr Arg Ala Met Ala Asp Gln Ala Arg Thr 350 355 360 365	1164
ATC CGC ATC CCG GTG CAC ATG GTC GAG GTG ATC AAC AAG CTG GGC CGC Ile Arg Ile Pro Val His Met Val Glu Val Ile Asn Lys Leu Gly Arg 370 375 380	1212
ATT CAA CGC GAG CTG CTG CAG GAC CTG GGC CGC GAG CCC ACG CCC GAG Ile Gln Arg Glu Leu Leu Gln Asp Leu Gly Arg Glu Pro Thr Pro Glu 385 390 395	1260
GAG CTG GCC AAA GAG ATG GAC ATC ACC CCG GAG AAG GTG CTG GAA ATC Glu Leu Ala Lys Glu Met Asp Ile Thr Pro Glu Lys Val Leu Glu Ile 400 405 410	1308
CAG CAA TAC GCC CGC GAG CCG ATC TCG TTG GAC CAG ACC ATC GGC GAC Gln Gln Tyr Ala Arg Glu Pro Ile Ser Leu Asp Gln Thr Ile Gly Asp 415 420 425	1356
GAG GGC GAC AGC CAG CTT GGC GAT TTC ATC GAA GAC AGC GAG GCG GTG Glu Gly Asp Ser Gln Leu Gly Asp Phe Ile Glu Asp Ser Glu Ala Val 430 435 440 445	1404
GTG GCC GTC GAC GCG GTG TCC TTC ACT TTG CTG CAG GAT CAA CTG CAG Val Ala Val Asp Ala Val Ser Phe Thr Leu Leu Gln Asp Gln Leu Gln 450 455 460	1452
TCG GTG CTG GAC ACG CTC TCC GAG CGT GAG GCG GGC GTG GTG CGG CTA Ser Val Leu Asp Thr Leu Ser Glu Arg Glu Ala Gly Val Val Arg Leu 465 470 475	1500
CGC TTC GGC CTT ACC GAC GGC CAG CCG CGC ACC CTT GAC GAG ATC GGC Arg Phe Gly Leu Thr Asp Gly Gln Pro Arg Thr Leu Asp Glu Ile Gly 480 485 490	1548
CAG GTC TAC GGC GTG ACC CGG GAA CGC ATC CGC CAG ATC GAA TCC AAG Gln Val Tyr Gly Val Thr Arg Glu Arg Ile Arg Gln Ile Glu Ser Lys 495 500 505	1596
ACT ATG TCG AAG TTG CGC CAT CCG AGC CGC TCA CAG GTC CTG CGC GAC Thr Met Ser Lys Leu Arg His Pro Ser Arg Ser Gln Val Leu Arg Asp 510 515 520 525	1644
TAC CTG GAC TGAGAGCGCC CGCCGAGGCG ACCAACGTAG CACGTGAGCC Tyr Leu Asp	1693
CCCAGCAGCT AGCCGCACCA TGGTCTCGTC C	1724

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 528 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Val Ala Ala Thr Lys Ala Ser Thr Ala Thr Asp Glu Pro Val Lys Arg  
1 5 10 15

-21-

Thr Ala Thr Lys Ser Pro Ala Ala Ser Ala Ser Gly Ala Lys Thr Gly  
                     20                    25                    30  
 Ala Lys Arg Thr Ala Ala Lys Ser Ala Ser Gly Ser Pro Pro Ala Lys  
                     35                    40                    45  
 Arg Ala Thr Lys Pro Ala Ala Arg Ser Val Lys Pro Ala Ser Ala Pro  
                     50                    55                    60  
 Gln Asp Thr Thr Thr Ser Thr Ile Pro Lys Arg Lys Thr Arg Ala Ala  
                     65                    70                    75                    80  
 Ala Lys Ser Ala Ala Ala Lys Ala Pro Ser Ala Arg Gly His Ala Thr  
                     85                    90                    95  
 Lys Pro Arg Ala Pro Lys Asp Ala Gln His Glu Ala Ala Thr Asp Pro  
                     100                    105                    110  
 Glu Asp Ala Leu Asp Ser Val Glu Glu Leu Asp Ala Glu Pro Asp Leu  
                     115                    120                    125  
 Asp Val Glu Pro Gly Glu Asp Leu Asp Leu Asp Ala Ala Asp Leu Asn  
                     130                    135                    140  
 Leu Asp Asp Leu Glu Asp Asp Val Ala Pro Asp Ala Asp Asp Asp Leu  
                     145                    150                    155                    160  
 Asp Ser Gly Asp Asp Glu Asp His Glu Asp Leu Glu Ala Glu Ala Ala  
                     165                    170                    175  
 Val Ala Pro Gly Gln Thr Ala Asp Asp Asp Glu Glu Ile Ala Glu Pro  
                     180                    185                    190  
 Thr Glu Lys Asp Lys Ala Ser Gly Asp Phe Val Trp Asp Glu Asp Glu  
                     195                    200                    205  
 Ser Glu Ala Leu Arg Gln Ala Arg Lys Asp Ala Glu Leu Thr Ala Ser  
                     210                    215                    220  
 Ala Asp Ser Val Arg Ala Tyr Leu Lys Gln Ile Gly Lys Val Ala Leu  
                     225                    230                    235                    240  
 Leu Asn Ala Glu Glu Glu Val Glu Leu Ala Lys Arg Ile Glu Ala Gly  
                     245                    250                    255  
 Leu Tyr Ala Thr Gln Leu Met Thr Glu Leu Ser Glu Arg Gly Glu Lys  
                     260                    265                    270  
 Leu Pro Ala Ala Gln Arg Arg Asp Met Met Trp Ile Cys Arg Asp Gly  
                     275                    280                    285  
 Asp Arg Ala Lys Asn His Leu Leu Glu Ala Asn Leu Arg Leu Val Val  
                     290                    295                    300  
 Ser Leu Ala Lys Arg Tyr Thr Gly Arg Gly Met Ala Phe Leu Asp Leu  
                     305                    310                    315                    320  
 Ile Gln Glu Gly Asn Leu Gly Leu Ile Arg Ala Val Glu Lys Phe Asp  
                     325                    330                    335  
 Tyr Thr Lys Gly Tyr Lys Phe Ser Thr Tyr Ala Thr Trp Trp Ile Arg  
                     340                    345                    350  
 Gln Ala Ile Thr Arg Ala Met Ala Asp Gln Ala Arg Thr Ile Arg Ile  
                     355                    360                    365  
 Pro Val His Met Val Glu Val Ile Asn Lys Leu Gly Arg Ile Gln Arg  
                     370                    375                    380

-22-

Glu Leu Leu Gln Asp Leu Gly Arg Glu Pro Thr Pro Glu Glu Leu Ala  
 385 390 395 400  
 Lys Glu Met Asp Ile Thr Pro Glu Lys Val Leu Glu Ile Gln Gln Tyr  
 405 410 415  
 Ala Arg Glu Pro Ile Ser Leu Asp Gln Thr Ile Gly Asp Glu Gly Asp  
 420 425 430  
 Ser Gln Leu Gly Asp Phe Ile Glu Asp Ser Glu Ala Val Val Ala Val  
 435 440 445  
 Asp Ala Val Ser Phe Thr Leu Leu Gln Asp Gln Leu Gln Ser Val Leu  
 450 455 460  
 Asp Thr Leu Ser Glu Arg Glu Ala Gly Val Val Arg Leu Arg Phe Gly  
 465 470 475 480  
 Leu Thr Asp Gly Gln Pro Arg Thr Leu Asp Glu Ile Gly Gln Val Tyr  
 485 490 495  
 Gly Val Thr Arg Glu Arg Ile Arg Gln Ile Glu Ser Lys Thr Met Ser  
 500 505 510  
 Lys Leu Arg His Pro Ser Arg Ser Gln Val Leu Arg Asp Tyr Leu Asp  
 515 520 525

## (2) INFORMATION FOR SEQ ID NO: 3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1508 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Mycobacterium tuberculosis
- (C) INDIVIDUAL ISOLATE: atcc27294

## (vii) IMMEDIATE SOURCE:

- (B) CLONE: pARC 8176

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 325..1293

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ACCAGCCCGA CGACCGACGA ACCCCGCCGC TTCGACGTGC CCAGCCGGCG CATCCCGCTG	60
TTCCCGACCG CGAACGGCCC GCACTCGAGC CGACGGCGAC AGCCGGCAAG AAGCGGTCAG	120
CCCGCGGGGA TTCGCCGACC ACGGTTAGCC GTCTGTTGGC CGCGGTTCCG GGTGTGCGCC	180
ACTGGCCACA CTTCTCAGGA CTTTCTCAGG TCTTCGGCAG ATTCTGCAC GTCACAGGGC	240
GTCAGATCAC TGCTGGGTGG GAACTCAAAG TCCGGCTTTG TCGTTAAACC CTGACAGTGC	300
AAGCCGATCG GGGAACGGCT CGCT ATG GCC GAT GCA CCC ACA AGG GCC ACC	351
Met Ala Asp Ala Pro Thr Arg Ala Thr	
530 535	



-23-

ACA AGC CGG GTT GAC ACA GAT CTG GAT GCT CAA AGC CCC GCG GCG GAC Thr Ser Arg Val Asp Thr Asp Leu Asp Ala Gln Ser Pro Ala Ala Asp 540 545 550	399
CTC GTG CGC GTC TAT CTG AAC GGC ATC GGC AAG ACG GCG TTG CTC AAC Leu Val Arg Val Tyr Leu Asn Gly Ile Gly Lys Thr Ala Leu Leu Asn 555 560 565	447
GCG GCG GAT GAA GTC GAA CTG GCC AAG CGC ATA GAA GCC GGG TTG TAT Ala Ala Asp Glu Val Glu Leu Ala Lys Arg Ile Glu Ala Gly Leu Tyr 570 575 580 585	495
GCC GAG CAT CTG CTG GAA ACC CGG AAG CGC CTC GGC GAG AAC CGA AAA Ala Glu His Leu Leu Glu Thr Arg Lys Arg Leu Gly Glu Asn Arg Lys 590 595 600	543
CGC GAC CTG GCG GCC GTG GTG CGT GAT GGC GAG GCC GCC CGC CGC CAC Arg Asp Leu Ala Ala Val Val Arg Asp Gly Glu Ala Ala Arg Arg His 605 610 615	591
CTG CTG GAA GCA AAC CTG CGG CTG GTG GTA TCG CTG GCC AAG CGC TAC Leu Leu Glu Ala Asn Leu Arg Leu Val Val Ser Leu Ala Lys Arg Tyr 620 625 630	639
ACG GGT CGG GGC ATG CCG TTG CTG GAC CTC ATC CAG GAG GGC AAC CTG Thr Gly Arg Gly Met Pro Leu Leu Asp Leu Ile Gln Glu Gly Asn Leu 635 640 645	687
GGT CTG ATC CGA GCG ATG GAG AAG TTC GAC TAC ACA AAG GGA TTC AAG Gly Leu Ile Arg Ala Met Glu Lys Phe Asp Tyr Thr Lys Gly Phe Lys 650 655 660 665	735
TTC TCA ACG TAT GCC ACG TGG TGG ATC CGC CAG GCC ATC ACC CGC GGA Phe Ser Thr Tyr Ala Thr Trp Trp Ile Arg Gln Ala Ile Thr Arg Gly 670 675 680	783
ATG GCC GAC CAG AGC CGC ACC ATC CGC CTG CCC GTA CAC CTG GTT GAG Met Ala Asp Gln Ser Arg Thr Ile Arg Leu Pro Val His Leu Val Glu 685 690 695	831
CAG GTC AAC AAG CTG GCG CGG ATC AAG CGG GAG ATG CAC CAG CAT CTG Gln Val Asn Lys Leu Ala Arg Ile Lys Arg Glu Met His Gln His Leu 700 705 710	879
GGT CGC GAA CGC ACC GAT GAG GAG CTC GCC GCC GAA TCC GGC ATT CCA Gly Arg Glu Arg Thr Asp Glu Glu Leu Ala Ala Glu Ser Gly Ile Pro 715 720 725	927
ATC GAC AAG ATC AAC GAC CTG CTG GAA CAC AGT CGC GAC CCG GTG AGT Ile Asp Lys Ile Asn Asp Leu Leu Glu His Ser Arg Asp Pro Val Ser 730 735 740 745	975
CTG GAT ATG CCG GTC GGC TCC GAG GAG GAG GCC CCT TTG GGC GAT TTC Leu Asp Met Pro Val Gly Ser Glu Glu Glu Ala Pro Leu Gly Asp Phe 750 755 760	1023
ATC GAG GAC GCC GAA GCC ATG TCC GCG GAG AAC GCG GTC ATC GCC GAA Ile Glu Asp Ala Glu Ala Met Ser Ala Glu Asn Ala Val Ile Ala Glu 765 770 775	1071
CTG TTA CAC ACC GAC ATC CGC AGC GTG CTG GCC ACT CTC GAC GAG CGT Leu Leu His Thr Asp Ile Arg Ser Val Leu Ala Thr Leu Asp Glu Arg 780 785 790	1119
GAC GAC CAG GTG ATC CCG CTG CGC TTC GGC CTG GAT GAC GGC CAA CCA Asp Asp Gln Val Ile Arg Leu Arg Phe Gly Leu Asp Asp Gly Gln Pro 795 800 805	1167

-24-

CGC ACC CTG GAT CAA ATC GGC AAA CTA TTC GGG CTG TCC CGT GAG CGG Arg Thr Leu Asp Gln Ile Gly Lys Leu Phe Gly Leu Ser Arg Glu Arg 810 815 820 825	1215
GTT CGT CAG ATC GAG CGC GAC GTG ATG AGT AAG CTG CGG CAC GGT GAG Val Arg Gln Ile Glu Arg Asp Val Met Ser Lys Leu Arg His Gly Glu 830 835 840	1263
CGG GCG GAT CGG CTG CGG TCG TAC GCC AGC TGAAGCTGGA CATCCTGAGC Arg Ala Asp Arg Leu Arg Ser Tyr Ala Ser 845 850	1313
CAGGTAGCAG ACGGTATGCC CGCCGCGCCA GCATAGCCTG CGGTGGGGCG GCGGGCAACC	1373
ATTTTCGCAG CTGGCCAAGT GTAGACTCAG CTGCAATGGA GGGTGCTGAA TGAACGAGTT	1433
GGTTGATACC ACCGAGATGT ACCTGCGGAC CATCTACGAC CTCGAGGAAG AGGGCGTGAC	1493
GCACTGCGTG CCGGA	1508

## (2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 323 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met	Ala	Asp	Ala	Pro	Thr	Arg	Ala	Thr	Thr	Ser	Arg	Val	Asp	Thr	Asp	1	5	10	15
Leu	Asp	Ala	Gln	Ser	Pro	Ala	Ala	Asp	Leu	Val	Arg	Val	Tyr	Leu	Asn	20	25	30	
Gly	Ile	Gly	Lys	Thr	Ala	Leu	Leu	Asn	Ala	Ala	Asp	Glu	Val	Glu	Leu	35	40	45	
Ala	Lys	Arg	Ile	Glu	Ala	Gly	Leu	Tyr	Ala	Glu	His	Leu	Leu	Glu	Thr	50	55	60	
Arg	Lys	Arg	Leu	Gly	Glu	Asn	Arg	Lys	Arg	Asp	Leu	Ala	Ala	Val	Val	65	70	75	80
Arg	Asp	Gly	Glu	Ala	Ala	Arg	Arg	His	Leu	Leu	Glu	Ala	Asn	Leu	Arg	85	90	95	
Leu	Val	Val	Ser	Leu	Ala	Lys	Arg	Tyr	Thr	Gly	Arg	Gly	Met	Pro	Leu	100	105	110	
Leu	Asp	Leu	Ile	Gln	Glu	Gly	Asn	Leu	Gly	Leu	Ile	Arg	Ala	Met	Glu	115	120	125	
Lys	Phe	Asp	Tyr	Thr	Lys	Gly	Phe	Lys	Phe	Ser	Thr	Tyr	Ala	Thr	Trp	130	135	140	
Trp	Ile	Arg	Gln	Ala	Ile	Thr	Arg	Gly	Met	Ala	Asp	Gln	Ser	Arg	Thr	145	150	155	160
Ile	Arg	Leu	Pro	Val	His	Leu	Val	Glu	Gln	Val	Asn	Lys	Leu	Ala	Arg	165	170	175	
Ile	Lys	Arg	Glu	Met	His	Gln	His	Leu	Gly	Arg	Glu	Arg	Thr	Asp	Glu	180	185	190	

[illegible]

(A) LENGTH: 25 base pairs  
(B) TYPE: nucleic acid

-26-

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "PCR primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TTCCATGGGG TATGTGGCAG CGACC

25

- (2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 27 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "PCR primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GTACAGGCCA GCCTCGATCC GCTTGGC

27

- (2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 28 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "PCR primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TTTCATGGCC GATGCACCCA CAAGGGCC

28

- (2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 27 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "PCR primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CTTGAATTCA GCTGGCGTAC GACCGCA

27

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

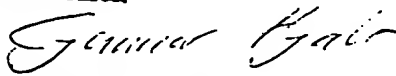
(PCT Rule 13bis)

<b>A. The indications made below relate to the microorganism referred to in the description</b> on page <u>17</u> , line <u>23</u>	
<b>B. IDENTIFICATION OF DEPOSIT</b> <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution The National Collections of Industrial and Marine Bacteria Limited (NCIMB)	
Address of depositary institution (including postal code and country)  23 St Machar Drive Aberdeen AB2 1RY Scotland, UK	
Date of deposit 15 June 1994	Accession Number NCIMB 40738
<b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
<p>In respect of all designated states in which such action is possible and to the extent that it is legally permissible under the law of the designated state, it is requested that a sample of the deposited micro-organism be made available only by the issue thereof to an independent expert, in accordance with the relevant patent legislation, e.g. Rule 28(4) EPC, and generally similar provisions <i>mutatis mutandis</i> for any other designated state.</p>	
<b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)	
<b>E. SEPARATE FURNISHING OF INDICATIONS</b> (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

<b>For receiving Office use only</b> <input checked="" type="checkbox"/> This sheet was received with the international application <div style="text-align: center; font-weight: bold; font-size: 1.2em;">12-03-1996</div> Authorized officer 	<b>For International Bureau use only</b> <input type="checkbox"/> This sheet was received by the International Bureau on:

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

<b>A.</b> The indications made below relate to the microorganism referred to in the description on page <u>17</u> , line <u>24</u>	
<b>B. IDENTIFICATION OF DEPOSIT</b> Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution <b>The National Collections of Industrial and Marine Bacteria Limited (NCIMB)</b>	
Address of depositary institution (including postal code and country)  <b>23 St Machar Drive Aberdeen AB2 1RY Scotland, UK</b>	
Date of deposit <b>15 June 1994</b>	Accession Number <b>NCIMB 40739</b>
<b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
In respect of all designated states in which such action is possible and to the extent that it is legally permissible under the law of the designated state, it is requested that a sample of the deposited micro-organism be made available only by the issue thereof to an independent expert, in accordance with the relevant patent legislation, e.g. Rule 28(4) EPC, and generally similar provisions <i>mutatis mutandis</i> for any other designated state.	
<b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)	
<b>E. SEPARATE FURNISHING OF INDICATIONS</b> (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
<div style="text-align: center;">For receiving Office use only</div> <div style="border: 1px solid black; padding: 5px;"><input checked="" type="checkbox"/> This sheet was received with the international application <b>12-03-1996</b></div> <div style="border: 1px solid black; padding: 5px;">Authorized officer </div>	<div style="text-align: center;">For International Bureau use only</div> <div style="border: 1px solid black; padding: 5px;"><input type="checkbox"/> This sheet was received by the International Bureau on:</div> <div style="border: 1px solid black; padding: 5px;">Authorized officer</div>

## CLAIMS

1. An isolated polypeptide which is a Group I sigma subunit of *Mycobacterium tuberculosis* RNA polymerase, or a functionally equivalent modified form thereof.  
5
2. A polypeptide according to claim 1 which amino acid sequence is identical to, or substantially similar to, SEQ ID NO: 2 or 4 in the Sequence Listing.  
10
3. An isolated nucleic acid molecule which has a nucleotide sequence coding for a polypeptide according to claim 1 or 2.
4. An isolated nucleic acid molecule selected from:  
15 (a) DNA molecules comprising a nucleotide sequence as shown in SEQ ID NO: 1 or SEQ ID NO: 3 encoding a Group I sigma subunit of *Mycobacterium tuberculosis* RNA polymerase;  
(b) nucleic acid molecules comprising a nucleotide sequence capable of hybridizing to a nucleotide sequence complementary the polypeptide coding region of a DNA molecule as defined in (a) and which codes for a polypeptide which is a Group I sigma subunit of *Mycobacterium tuberculosis* or a functionally equivalent modified form thereof; and  
20 (c) nucleic acid molecules comprising a nucleic acid sequence which is degenerate, as a result of the genetic code, to a nucleotide sequence as defined in (a) or (b) and which codes for a polypeptide which is a Group I sigma subunit of *Mycobacterium tuberculosis* or a functionally equivalent modified form thereof.  
25
5. A vector which comprises a nucleic acid molecule according to claim 3 or 4.  
30

6. A vector according to claim 5 which is the plasmid vector pARC 8175 (NCIMB 40738) or pARC 8176 (NCIMB 40739).
- 5 7. A vector according to claim 5 which is an expression vector capable of mediating the expression of a polypeptide according to claim 1 or 2.
8. A host cell harbouring a vector according to any one of claims 5 to 7.
- 10 9. A process for production of a polypeptide according to claim 1 or 2 which comprises culturing a host cell according to claim 8 transformed with an expression vector according to claim 7 under conditions whereby said polypeptide is produced and recovering said polypeptide.
- 15 10. A method of assaying for compounds which have the ability to inhibit the association of a sigma subunit with a *Mycobacterium tuberculosis* core RNA polymerase, said method comprising (i) contacting a compound to be tested for said inhibition ability with a  
20 polypeptide according to claim 1 or claim 2 and a *Mycobacterium tuberculosis* core RNA polymerase; and (ii) detecting whether the said polypeptide associates with the said core RNA polymerase to form RNA polymerase holoenzyme.
- 25 11. A method according to claim 10 wherein polypeptides which are associated to core RNA polymerase and / or polypeptides which are not associated to core RNA polymerase are detected by chromatography such as gel filtration.
- 30 12. A method according to claim 10 wherein RNA polymerase holoenzyme is detected by immunoprecipitation, using an antibody binding to RNA polymerase holoenzyme.



13. A method of assaying for compounds which have the ability to inhibit sigma subunit-dependent transcription by a *Mycobacterium tuberculosis* RNA polymerase, said method comprising (i) contacting a compound to be tested for said inhibition ability with a polypeptide according to claim 1 or claim 2, a *Mycobacterium tuberculosis* core RNA polymerase, and a DNA having a coding sequence operably-linked to a promoter sequence capable of recognition by said core RNA polymerase when bound to said polypeptide, said contacting being carried out under conditions suitable for transcription of said coding sequence when *Mycobacterium tuberculosis* RNA polymerase is bound to said promoter; and (ii) detecting formation of mRNA corresponding to said coding sequence.
14. A method of determining the protein structure of a *Mycobacterium tuberculosis* RNA polymerase sigma subunit, characterised in that a polypeptide according to claim 1 or claim 2 is utilized in X-ray crystallography.

1 / 1

Fig. 1

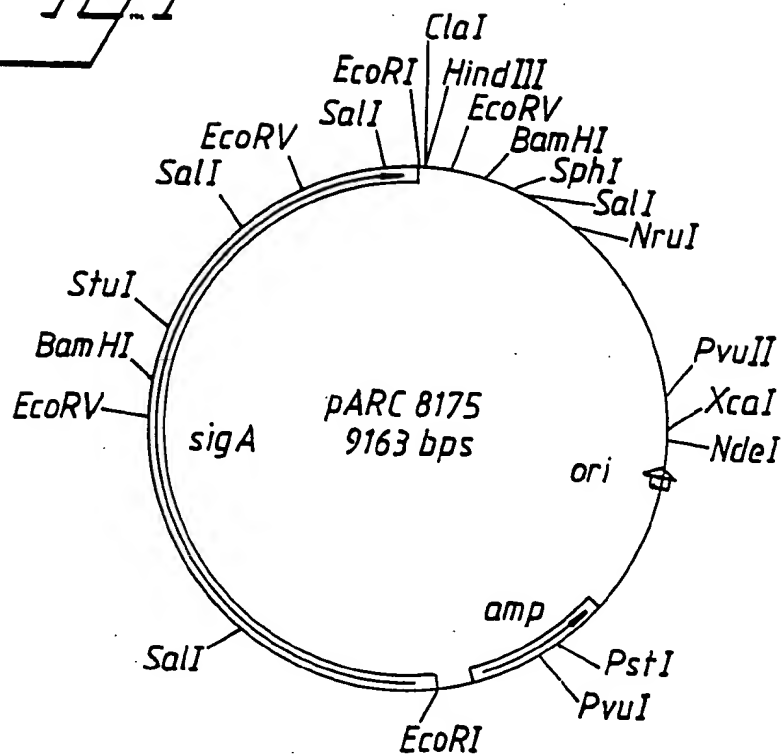
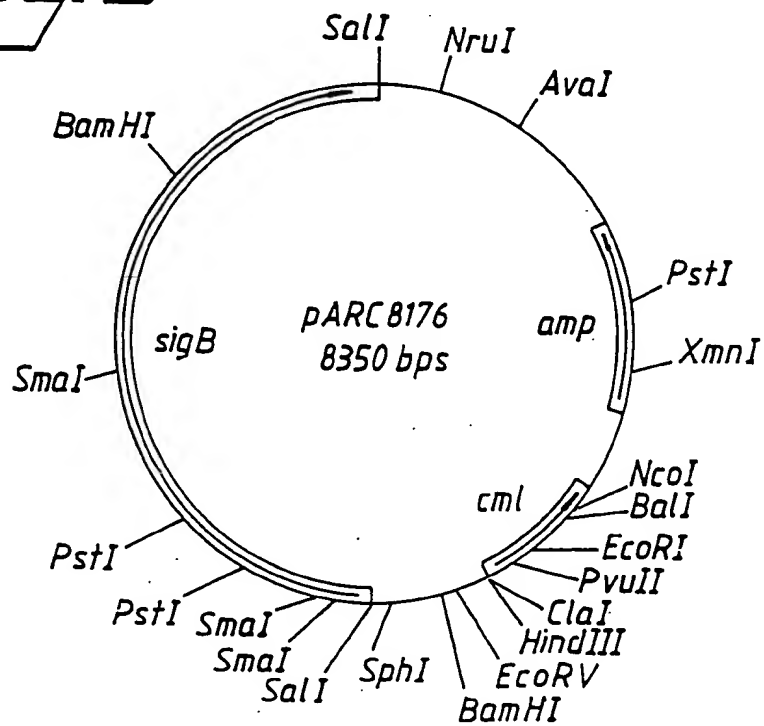


Fig. 2



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 96/00319

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/35 // G01N 033/53

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EDOC, MEDLINE, BIOSIS, DERWENT BIOTECH ABSTRACT, EMBL/GENBANK/DBJ

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 9517511 A2 (AGRESEARCH NEW ZEALAND PASTORAL AGRICULTURE RESEARCH INSTITUTE LTD), 29 June 1995 (29.06.95)  --	1-9
A	Abstracts of the general meeting of the American Society for Microbiology, Vol 94, 1994, D.M. Welty et al: "Identification of a putative rpoS homologue from M. marinum M. tuberculosis, M. ulcerans, and M. haemophilum", see page 177  --	1-9
A	Journal of Cellular Biochemistry Supplement, Vol 19B, 1995, T.S.Balganesh et al: "B3201 Sigma Factors of M.tuberculosis RNA Polymerase", page 73  --	1-9

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

## \* Special categories of cited documents

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

4 July 1996

Date of mailing of the international search report

24 -07- 1996

Name and mailing address of the ISA/

Swedish Patent Office

Box 5055, S-102 42 STOCKHOLM

Facsimile No. +46 8 666 02 86

Authorized officer

PATRICK ANDERSSON

Telephone No. +46 8 782 25 00

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/SE 96/00319

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Molecular Microbiology, Vol 15, No 2, 1995, Mima Predich et al: "Characterization of RNA polymerase and two sigma-factor genes from Mycobacterium smegmatis" page 355 - page 366  -- -----	1-9

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International application No.

01/04/96

PCT/SE 96/00319

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A2- 9517511	29/06/95	NONE	

**THIS PAGE BLANK (USPTO)**